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14. ABSTRACT MicroRNAs (miRNAs) are a class of small noncoding RNAs that control gene expression by targeting mRNAs and triggering either translational repression or RNA degradation. MiR-10b is one miRNA whose expression has been reported to be significantly deregulated in breast cancer. We have identified the VEGF receptor Flt-1 and guanine nucleotide exchange factor (GEF) Tiam1 as targets of miR-10b in breast cancer. We have focused on Tiam1 because it is a positive regulator of cell motility and invasion. Furthermore, Tiam1 expression increases with human breast cancer grade. We report here that downregulation of Tiam1 by miR-10b represses in vitro migration and invasion of breast tumor cells. Tiam1 is a GEF for Rac, a Rho-GTPase that regulates actin dynamics at the leading edge during cell movement. We report that miR-10b-induced downregulation of Tiam1 results in a corresponding decrease in Rac activation, thereby impairing cell motility. The ability of miR-10b to target Tiam1 provides a novel mechanism for regulation of Tiam1 in breast cancer.

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Introduction

MicroRNAs (miRNAs) are a class of small noncoding RNAs that control gene expression by targeting mRNAs and triggering either translational repression or mRNA degradation. Recent evidence has shown that miRNAs are aberrantly expressed in human cancer (Calin and Croce, 2006; Iorio et al., 2005; Lu et al., 2005; Ozen et al., 2008) and that they can affect key cell biological processes that affect tumor progression including migration, invasion, epithelial to mesenchymal transition (Burk et al., 2008; Korpal et al., 2008) and metastasis (Huang et al., 2008; Tavazoie et al., 2008; Zhu et al., 2008). The challenge ahead is to elucidate specific mechanisms by which miRNAs regulate such processes.

MiR-10b is one of 29 miRNAs whose expression has been reported to be significantly deregulated in breast cancer (Iorio et al., 2005). This miRNA achieved prominence because its expression in primary breast tumors was found to correlate with their ability to metastasize, and it was shown to promote the migration and invasion of breast carcinoma cells *in vitro* (Ma et al., 2007).This seminal role for miR-10b in breast cancer, however, was challenged recently based on the analysis of miR-10b expression in a large group of patients with early breast cancer (Gee et al., 2008). In this study, miR-10b expression did not correlate with development of distant metastases, recurrence-free survival or distant-relapse-free survival. Instead, miR-10b expression correlated inversely and significantly with tumor size, grade and vascular invasion. These data infer that miR-10b impedes specific functions associated with breast cancer progression, and they highlight the need for more mechanistic studies.

During this year of the fellowship, we analyed miR-10b function in breast carcinoma cells. Our data indicate that it suppresses their migration and invasion. We have previously identified the VEGF receptor Flt-1 as a novel miR-10b target. However, the function of Flt-1 in breast cancer is not yet known. To define a mechanism that accounts for the suppressive function of miR-10b on cell motility, we identified Rac guanine nucleotide exchange factor (GEF) Tiam1 as a second miR-10b target and demonstrated that miR-10b inhibits Tiam1-dependent Rac activation and migration/invasion. These data reveal a novel function of miRNAs and they support the conclusion from clinical data that miR-10b expression correlates inversely with breast cancer progression.

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To assess the ability of miR-10b to regulate migration, we used two breast carcinoma cell lines (SUM149PT and SUM159PT) that are highly motile and invasive, and one (T47D) that is not. RT-PCR analysis revealed that SUM149PT and SUM159PT cells lacked miR10b expression but T47D cells expressed it (Fig. 1A). To examine whether exogenous expression of miR-10b influences migration, we used a commercially available miR-10b precursor for *de novo* expression in SUM159PT cells. This precursor is a chemically-modified double-stranded RNA modeled on the sequence of mature miR-10b. For a control, we designed a miR-10b mutant with a single base pair substitution in the seed sequence of the mature strand (Fig. 1B).

By introducing mismatch into the critical seed region, binding of the miRNA to its target genes should be reduced or abolished. A non-targeting miRNA was used as an additional negative control. Transient expression of miR-10b in SUM159PT cells resulted in expression of mature miR-10b, as assessed by RT-PCR. The miR-10b mutant, differing from miR-10b by a single base pair, is also detected by the primers used but is amplified with lower fidelity. Expression of miR-10b resulted in a 2-fold decrease in both migration and invasion as compared to non-targeting and mutant controls (Fig. 1C). To confirm this result, miR-10b was expressed in the less motile SUM149PT cells, which resulted in a 3-fold decrease in cell migration (Fig. 1D).

To confirm that miR-10b inhibits cell motility and that we were not observing an artifact of the mimic, we used a miR-10b expression vector. This retroviral vector encodes the genomic sequence of the human *miR-10b* gene and requires that mature miR-10b be generated through endogenous cellular processing. We used this vector to express miR-10b in SUM159PT cells and confirmed expression of the mature sequence by RT-PCR (Fig. 1E). Ectopic expression of miR-10b resulted in a significant decrease in cell migration and invasion in comparison to the empty vector (Fig. 1E).

We next asked whether inhibition of endogenous miR-10b in T47D cells would affect their migration. For this purpose, we designed an antisense oligonucleotide to silence miR-10b. Indeed, expression of this antisense oligonucleotide inhibited the migration of T47D cells in a concentration-dependent manner with maximal and significant inhibition observed at 20nM (Fig. 1F).

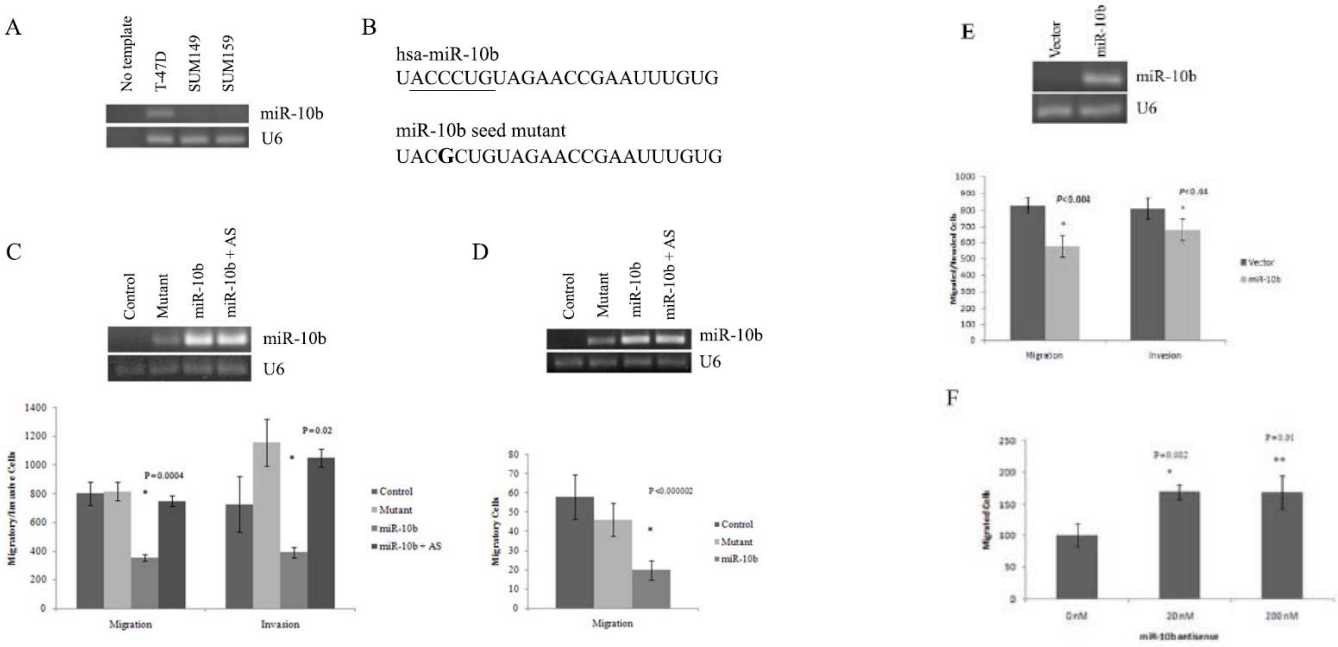

Figure 1. miR-10b suppressed breast cancer cell migration and invasion

To understand the mechanisms by which miR-10b represses cell motility, we used computational algorithms to help identify miR-10b target genes. The search program TargetScan revealed several predicted targets known to play a role in cell migration and invasion, including T lymphoma invasion and metastasis 1 (Tiam1) and nuclear factor of activated T cells 5 (NFAT5). Tiam1 was of particular interest because its expression correlates with epithelial tumorigenicity, the metastatic potential of human breast cancer cell lines (Minard et al., 2004), and increased breast cancer grade (Adam et al., 2001). The predicted target site for miR-10b is a single 8mer site, comprised of the seed match flanked by both the match at position 8 and the A at position 1 (Lewis et al., 2005). We observed a dramatic reduction in Tiam1 protein levels in both SUM159PT and SUM149PT cells expressing miR-10b, as compared to controls (Fig. 2A and 2B). Co-transfection of the miR-10b mimic with miR-10b antisense rescued expression of Tiam1. Conversely, transfection of miR-10b antisense in T47D cells to silence endogenous miR-10b led to a corresponding increase in Tiam1 protein (Fig. 2C).

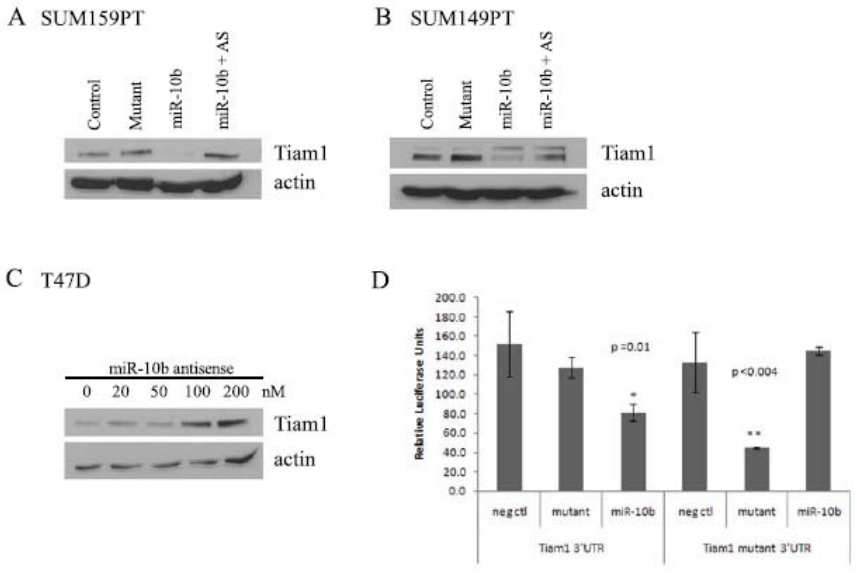

Figure 2. miR-10b regulates expression of Tiam1.

To determine whether regulation of Tiam1 expression of miR-10b is direct, we utilized a luciferase reporter gene fused to the wild-type Tiam1 3'UTR. Expression of miR-10b reduced the activity of luciferase while a miR-10b seed mutant had no effect, indicating that miR-10b targets Tiam1 directly (Fig. 2D). As a control, we developed a second luciferase reporter with a single base pair mutation in the Tiam1 3'UTR, at the site corresponding to the miR-10b seed mutant. As expected, miR-10b had no effect on the luciferase activity of this reporter, whereas the miR-10b seed mutant, a perfect match in the seed region, repressed the luciferase signal.

Next, we asked whether Tiam1 down-regulation is responsible for inhibition of cell motility by miR-10b. To determine whether SUM159PT cells are dependent on Tiam1 for cell motility, we diminished Tiam1 expression in these cells using a Tiam1 siRNA pool (Fig. 3A). Knockdown of Tiam1 resulted in a 40% decrease in both cell migration and cell invasion, similar to the change seen with *de novo* expression of miR-10b. Importantly, co-transfection of miR-10b and Tiam1 cDNA lacking the 3'UTR was able to rescue miR-10b-induced repression of cell motility (Fig. 3B), suggesting that Tiam1 is the factor responsible for decreased cell motility in cells expressing miR-10b.

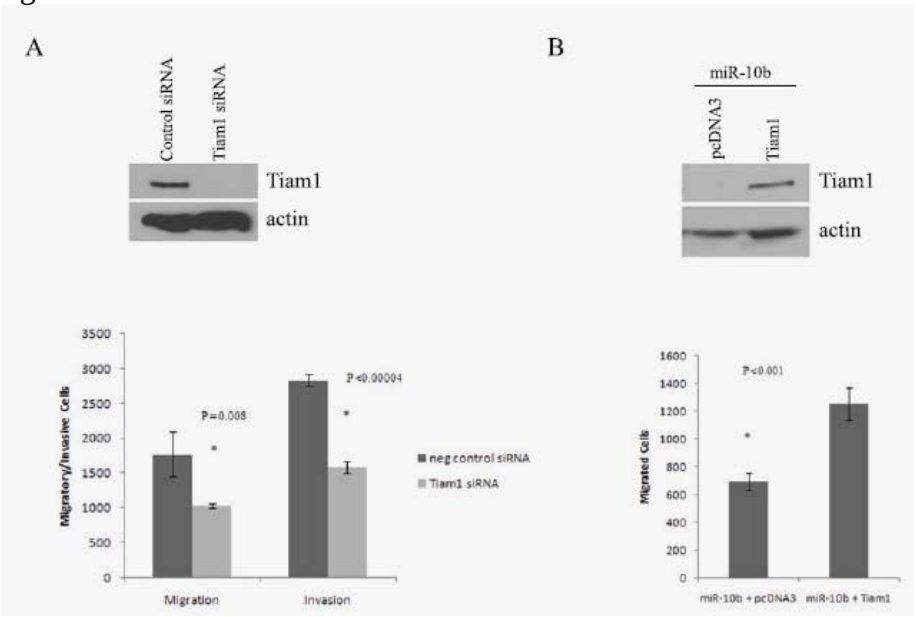

Figure 3. Exogenous expression of Tiam1 rescues cell migration.

Tiam1 is a guanine nucleotide exchange factor for Rac, a Rho-GTPase which regulates actin dynamics at the leading edge during cell movement. We hypothesized that miR-10b-induced downregulation of Tiam1 results in a corresponding decrease in Rac activation, thereby impairing cell motility. Knockdown of Tiam1 in SUM159PT cells resulted in a 50% decrease in Rac activation (Fig. 4A), indicating that Tiam1 expression is necessary for optimal Rac activation. Similarly, *de novo* expression of miR-10b in this cell line also repressed activation of Rac (Fig. 4B).

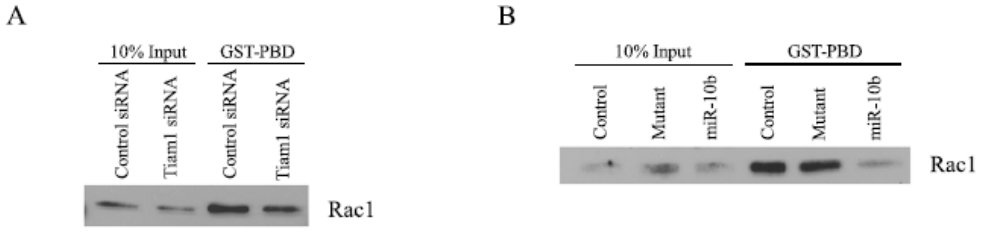

Figure 4. miR-10b represses Tiam1 dependent activation of Rac1.

Key Research Accomplishments

- miR-10b suppresses breast carcinoma migration and invasion
- miR-10b regulates expression of GEF Tiam1
- miR-10b represses Tiam1-dependent activation of Rac1
- Presentation of work at lab meetings and departmental data club
- Presentation of work at UMass Memorial's monthly Breast Cancer Conference
- Poster presentation at the 2007 Era of Hope Meeting

Reportable Outcomes

None

Conclusion

An important conclusion drawn from our data is that Tiam1-mediated Rac activation and migration/invasion can be regulated by a specific miRNA. Although it is known that Tiam1 expression increases with breast cancer grade, little is known about how this GEF is regulated in breast tumors. The ability of miR-10b to target Tiam1 provides one such mechanism, which is substantiated by the observation that miR-10b expression decreases as a function of grade in breast cancer.

Our data contrast markedly with the data reported by Ma et al. (Ma et al., 2007), who concluded that miR-10b promotes the migration and invasion of breast carcinoma cells by a mechanism that involves the HoxD10 induction of RhoC expression. Given that we used similar cell lines (e.g., SUM149PT) and experimental conditions, it is difficult to reconcile this opposing conclusion of miR-10b function in breast cancer. Our mechanistic data, however, mesh with the recent observation that miR-10b expression in human breast tumors correlates inversely with a more invasive phenotype as indicated by tumor stage, grade and vascular invasion. We also question the purported role of miR-10b in inducing RhoC expression because we had reported previously that SUM149PT cells, which were shown to lack miR-10b expression by both us (Fig. 1) and Ma et al, express relatively high levels of RhoC.

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